FEBS Letters 588 (2014) 436–442

journal homepage: www.FEBSLetters.org

Construction of carotenoid biosynthetic pathways using squalene synthase

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ARTICLE INFO

Article history:
Received 26 October 2013
Revised 28 November 2013
Accepted 3 December 2013
Available online 10 December 2013

Edited by Richard Cogdell

Keywords:
Squalene
Carotenoid
Desaturase
Lycopersene
Chimeric pathway

ABSTRACT

The first committed steps of steroid/hopanoid pathways involve squalene synthase (SQS). Here, we report the *Escherichia coli* production of diaponeurosporene and diapolycopene, yellow C_{30} carotenoid pigments, by expressing human SQS and *Staphylococcus aureus* dehydrosqualene (C_{30} carotenoid) desaturase (CrtN). We suggest that the carotenoid pigments are synthesized mainly via the desaturation of squalene rather than the direct synthesis of dehydrosqualene through the non-reductive condensation of prenyl diphosphate precursors, indicating the possible existence of a "squalene route" and a "lycopersene route" for C_{30} and C_{40} carotenoids, respectively. Additionally, this finding yields a new method of colorimetric screening for the cellular activity of squalene synthases, which are major targets for cholesterol-lowering drugs.
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1. Introduction

Steroids/hopanoids and carotenoids constitute two of the major sub-groups of isoprenoids. Despite the large differences in the structures of the end products, these two groups share a branch point and chemistry in the first committed step (Fig. 1). The first committed step in steroid/hopanoid pathways is the biosynthesis of squalene from two farnesyl diphosphates (FPPs), which is catalyzed by squalene synthase (SQS). The first step in carotenoid pathways is the biosynthesis of either phytoene (C_{40}) from two geranylgeranyl diphosphates (GGPPs) or diapophytoene (C_{30}, also called dehydrosqualene) from two FPPs. The difference in the end-product structures is the presence or absence of the double bond in the center of the backbone (Fig. 1). Because they are placed at the branching position, these enzyme activities determine which pathway the precursors (FPP or GGPP) enter. Once formed, squalene is exclusively used to generate steroids/hopanoids, whereas dehydrosqualene/phytoene are used to generate carotenoid pigments.

The reaction catalyzed by SQS and C_{30} carotenoid synthases are similar with regard to their 2-step catalytic mechanisms. The first half-reaction catalyzed by SQS is identical to that of carotenoid synthases (Fig. 1). The difference is in the second half-reaction: SQS converts the intermediate presqualene diphosphate (PSPP) to squalene using NADPH as a cofactor, whereas carotenoid synthases convert the intermediate without NADPH to make dehydrosqualene (C_{40} carotenoid synthases work in exactly the same way except they use GGPPs as substrates). Here, *in vitro* experiments showed that SQS could synthesize detectable amounts of dehydrosqualene in the absence of NADPH [1–4]. Thus, SQS could generate the carotenoid dehydrosqualene, and this could be a theoretically possible route for carotenoid biosynthesis.

Another possible route for carotenoid production is the direct conversion of squalene into carotenoids (Fig. 1). Based on various indirect observations, such as detection of the C_{40} squalene counterpart lycopersene in carotenogenic organisms [5], this route was once believed to be the *bona fide* biosynthetic route for carotenoids. The existence of this route was later refuted by the discovery of a series of specialized carotenoid synthases from various carotenogenic organisms and is now only discussed in historical statements as the "lycopersene problem" [5].

In this study, we report the *Escherichia coli* production of diaponeurosporene and diapolycopene, yellow C_{30} carotenoid pigments, by expressing human SQS and *Staphylococcus aureus* dehydrosqualene desaturase (CrtN, also called as diapophytoene desaturase). Time-course analysis of product accumulation,

Abbreviations: SQS, squalene synthase; hSQS, human squalene synthase; CrtN, dehydrosqualene desaturase; CrtM, dehydrosqualene synthase; CrtI, phytoene desaturase; CrtB, phytoene synthase; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; PSPP, presqualene diphosphate

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http://dx.doi.org/10.1016/j.febslet.2013.12.003

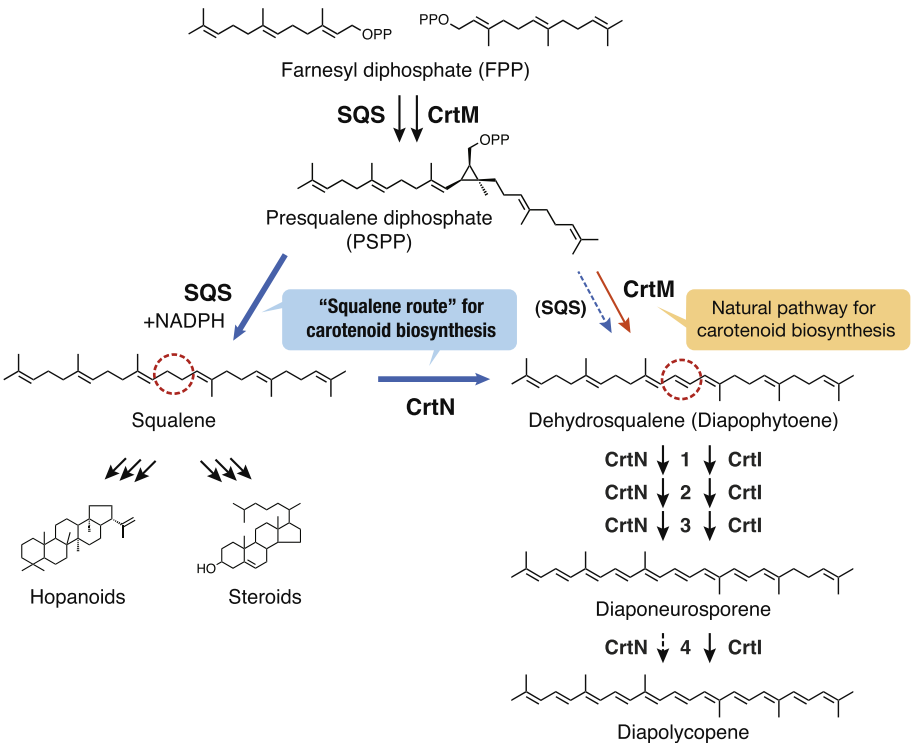

Fig. 1. Squalene and carotenoid biosynthetic pathway. SQS synthesizes squalene using NADPH, and CrtM synthesizes dehydrosqualene. The structural difference between squalene and dehydrosqualene is the presence of absence of double bonds, as indicated in red circles. In the absence of NADPH, SQS could directly produce dehydrosqualene via non-reductive rearrangement of presqualene diphosphate (PSPP), as indicated in a dashed blue arrow. Alternatively, in this study, we suggest that squalene can be desaturated by CrtN to make dehydrosqualene ("squalene route"), emphasized by thick blue arrow. Either way, the resulting dehydrosqualene would be further desaturated in 3- or 4-steps, yielding the C_{30} carotenoid pigments diaponeurosporene or diapolycopene, respectively. See Supplementary Fig. 1 for C_{40} equivalent pathways ("lycopersene route" and natural "phytoene route").

together with comparison of a co-expression system employing another desaturase, *Pantoea ananatis* CrtI [6], indicates that CrtN likely directly desaturates squalene to make dehydrosqualene and subsequent C_{30} carotenoid pigments. This finding revives the idea of the possible existence of a "squalene route" and "lycopersene route" for C_{30} and C_{40} carotenoid pigments, respectively (Fig. 1 and Supplementary Fig. 1). Additionally, our finding potentially provides new means of high-throughput colorimetric screening of the cellular activity of squalene synthases, an important target for cholesterol-lowering drugs.

2. Materials and methods

2.1. Bacterial strains and genetic manipulations

E. coli XL10-Gold Kan^R (Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*) *173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r) Tn*5* (Kan^r) Amy]) (Stratagene, La Jolla, CA) was used for DNA cloning, while XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]) (Stratagene) was used for the carotenoid or squalene production analysis. Polymerase chain reaction (PCR) amplifications were performed using *vent* polymerase (New England BioLabs, Ipswich, MA). Restriction enzymes and DNA modifying enzymes were purchased from New England BioLabs. DNA was purified using the Zymo DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA). Chemical competent cells were prepared using the Z-Competent™ *E. coli* Transformation Kit & Buffer Set (Zymo Research). Plasmid DNA was prepared with the Plasmid Miniprep Kit from Sigma–Aldrich. The nucleotide sequences were confirmed with the BigDye® Terminator v3.1 Cycle Sequencing Kit and analyzed with a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.2. Plasmids

pUC-*hsqs* was constructed by truncating 30 residues of the N-terminus and 47 residues of the C-terminus of the human squalene synthase gene (*hsqs*), using pHSS16 [7] as a template, and inserting the sequence into the *Xba*I/*Xho*I site of pUC18m [8]. A P*lac-hsqs* fragment was PCR-amplified from pUC-*hsqs* and cloned into the *Bam*HI site of pACmod to make pAC-*hsqs*. pAC-*crtM* was constructed by inserting the *E. coli* codon-optimized *crtM* gene from *S. aureus* (purchased from DNA 2.0 Inc., Menlo Park, CA) into the *Xba*I/*Xho*I restriction site of pAC-*hsqs*. pUCara was constructed by replacing the *lac* promoter region of pUC18m [8] with the P_C–*araC* and P_{BAD} promoters derived from pBAD32 (Invitrogen, Carlsbad, CA). pUCara-*crtN* was constructed by inserting the *S. aureus crtN* gene from pAC-*crtN* [9] into the *Xho*I/*Apa*I site of pUCara. pUCara-*crtI* was constructed by inserting the *P. ananatis crtI* gene from pAC-*crtE-crtI* [9] into the *Xho*I/*Apa*I site of pUCara.

2.3. Pigment formation analysis

Plasmids were transformed into XL1-Blue, and the transformants were plated onto LB-Lennox agar plates containing 50 μg/mL carbenicillin (carb) and 30 μg/mL chloramphenicol (cm) to form colonies. These colonies were picked and inoculated into 500 μL LB-Lennox (carb/cm) medium in a 96-deep-well plate and cultured at 37 °C, 1000 rpm, for 16 h. An aliquot (40 μL) of these pre-cultures was transferred to 2 mL Terrific Broth (TB) (carb/cm) in a 48-deep-well plate and cultured at 30 °C, 1000 rpm, for 48 h. Cells were harvested, washed with saline, and centrifuged to obtain cell pellets; the supernatants were discarded. After brief vortexing, 1 mL acetone was added to each of the cell pellets, and they were immediately vortexed for 1 min to extract carotenoids, followed by centrifugation.

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